Hidden chromosome abnormalities in haematological malignancies detected by multicolour spectral karyotyping

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Cytogenetic analysis provides critical information of diagnostic and prognostic importance for haematological malignancies[1,2]. In fact, the identification of recurring chromosomal breakpoints in leukaemias and lymphomas has expedited the cloning of genes whose translocation-induced deregulation causes malignant transformation[3,4]. The pillar of karyotype analysis rests on chromosome banding techniques that have the distinct advantage that the entire genome can be analysed in a single experiment. However, poorly spread or contracted metaphase chromosomes and highly rearranged karyotypes with numerous marker chromosomes, common in tumour cell preparations, are often difficult to interpret unambiguously and subtle chromosomal aberrations, in particular the exchange of telomeric chromatin or small insertions remain elusive. Fluorescence *in situ* hybridization (FISH) overcomes some of these limitations, but is mainly utilized to confirm the presence of previously characterized or suspected aberrations[5]. We have developed a novel approach, termed spectral karyotyping or SKY[6,7] based on the hybridization of 24 fluorescently labelled chromosome painting probes that allows the simultaneous and differential colour display of all human chromosomes. We have used SKY to complement conventional banding techniques in haematological malignancies by analysing 15 cases with unidentified chromosome aberrations. In all instances SKY provided additional cytogenetic information, including the identification of marker chromosomes, the detection of subtle chromosomal translocations and the clarification of complex chromosomal rearrangements. Thus, SKY in combination with standard chromosome banding allows the characterization of chromosomal aberrations in leukaemia with unprecedented accuracy.

We were successful in using SKY as a novel molecular cytogenetic screening test for chromosomal aberrations to an extended series of bone marrow samples from patients with acute and chronic leukaemias, myelodysplastic syndromes and lymphomas (Table 1). An example is presented for a case of chronic myeloid leukaemia (CML, 20818) in Fig. 1. Several chromosomal aberrations were detected by means of G-banding and the karyotype was interpreted as 46,XX,t(9;22)(q34;q11),add(11)(q23.3),t(14;15)(q22;q13) (Fig. 1*a*). However the origin of the chromosomal material translocated to chromosome 11 could not be determined by G-banding alone. The hybridization of the 24 differentially labelled human chromosome painting probes to metaphase preparations from this case is presented in a red-green-blue (RGB)-display (see Methods) which permits the analysis of important parameters of the hybridization such as signal brightness and painting homogeneity (Fig. 1*b*). The strong hybridization intensities indicate that archived cell pellets can be used successfully for SKY analysis. The metaphase chromosomes were rekaryotyped based on the spectral classification (Fig. 1*c*). SKY confirmed all aberrations detected by G-banding and in addition identified the origin and the chromosomal mechanism of the aberration involving chromosomes 3 and 11 as a reciprocal translocation t(3;11)(q26;q23.3). In 7 of the 15 cases (21232, 21201, 21500, 21646, 21571, 21050, 20955), the aberrations detected by SKY were confirmed using conventional dual-colour or triple-colour FISH with chromosome painting probes (not shown).

Twelve of the 15 patients had aberrant chromosomes with unidentified material attached to an identifiable chromosome. In each case the unidentified material could be defined unambiguously by SKY. In three cases the aberrations were shown to be deletions (21272, 21500 and 20801) and in two others they appeared

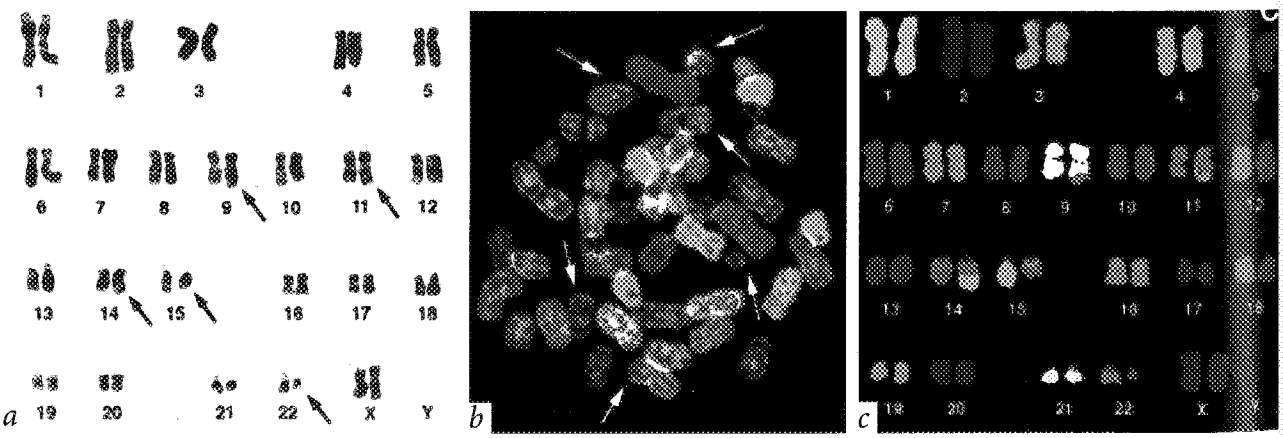

Fig. 1 Comprehensive cytogenetic analysis of a case of chronic myeloid leukaemia (20818). ***a***, G-banding analysis, karyotype. The aberrant chromosomes are denoted by arrows. The karyotype was interpreted as 46,XX,t(9;22)(q34;q11),add(11)(q23.3),t(14;15)(q22;q13). ***b***, Spectral karyotyping of metaphase chromosomes of the same case as shown in (*a*). Chromosome spectra were visualized by assigning a RGB look up table to different regions of the spectrum (see Methods). Aberrant chromosomes are highlighted by arrows. Aberrations were detected that involve chromosomes 3, 9, 11, 14, 15, and 22. ***c***, Spectral karyotype of the metaphase cell shown in (*b*) after spectra based classification. Chromosomes were assigned a pseudocolour according to the measured spectrum. The combination of banding and SKY allowed us to refine the karyotype to 46,XX,t(9;22)(q34;q11),t(3;11)(q26;q23.3),t(14;15)(q22;q13). The translocation of chromosome 11 material to the long arm of chromosome 3 was not detectable by G-banding analysis.

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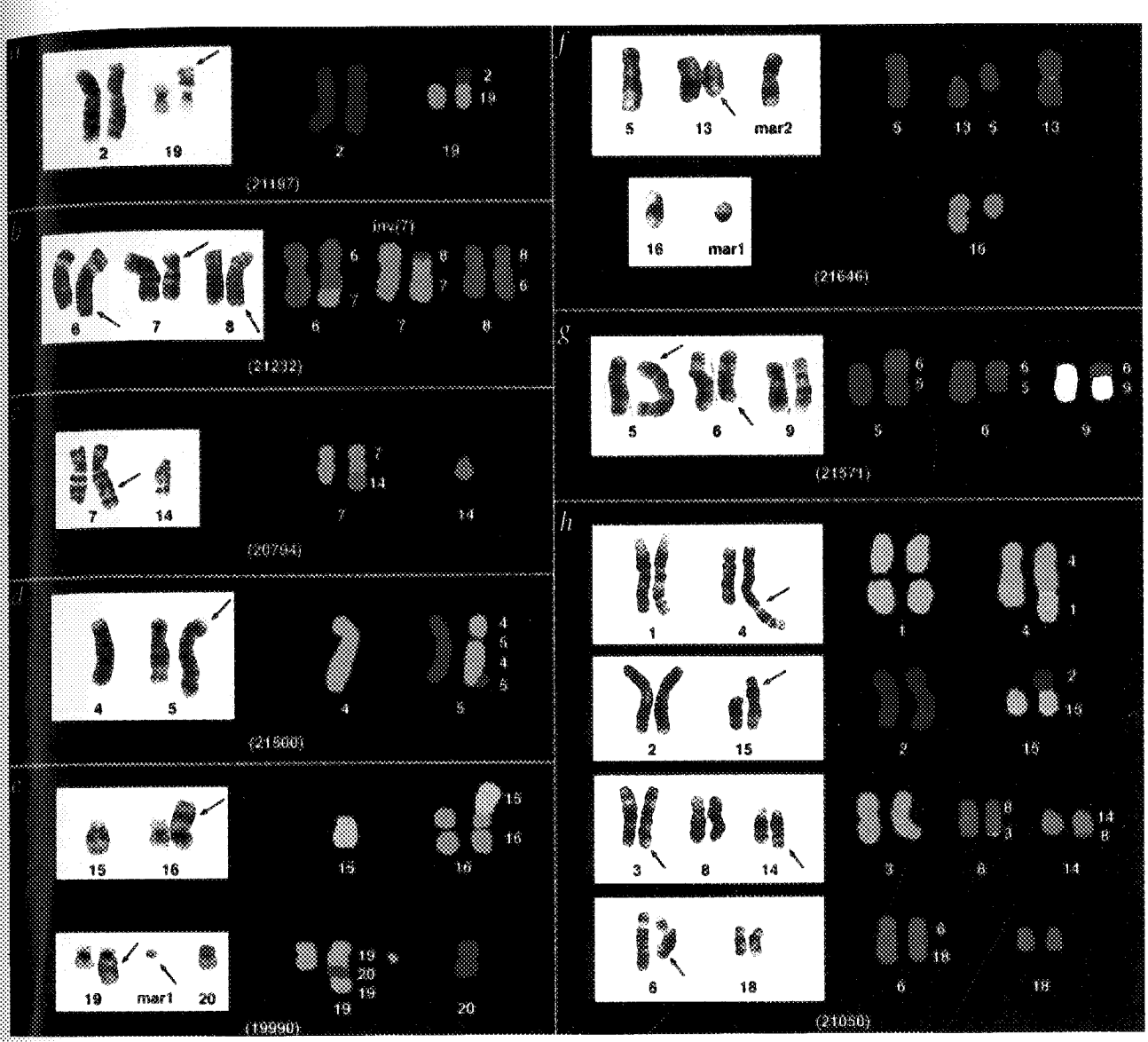

Fig. 2 Visualization of aberrant chromosomes after G-banding and SKY from cases 21197, 21232, 20794, 21500, 19990, 21646, 21571 and 21050. The aberrant chromosomes and their normal homologues are displayed. Numbers below the chromosomes identify the non-involved normal homologues. The numbers on the right side of the marker chromosomes denote the chromosomal origin of translocated material. The cytogenetic interpretation of the chromosomal aberrations is summarized in Table 1. ***a***, Previously unidentified material on a der(19) could be identified as chromosome 2. ***b***, SKY confirmed the presence of a complex three-way translocation involving chromosome 6, 7 and 8. ***c***, The breakpoint on chromosome 7 could be refined to chromosome band 7q22. ***d***, Karyotype interpretation was modified after SKY. The monosomy 5 was shown to be in fact a monosomy 4. ***e***, An add(16) could be specified to be a der(16)t(15;16), and a der(19) was identified as a complex chromosomal rearrangement involving chromosome 19 and 20. Also, a small marker was shown to be derived from chromosome 19. ***f***, Identification of a del(13) as a del(5), and clarification of marker 1 as being derived from chromosome 16, and marker 2 as being derived from chromosome 13. ***g***, Identification of a reciprocal translocation t(5;6). In this case a 'normal' chromosome 9 was shown to be a translocation der(9)t(6;9). ***h***, Confirmation of a t(1;4). Characterization of a t(2;15). Identification of a 'normal' chromosome 8 as a der(8)t(3;8), and refinement of a del(6) indicating a der(6)t(6;18).

to be duplications (21571 and 21114). Identification of aberrant chromosomes from eight of these cases is shown in Fig. 2. Two patients with CML had an add(19)(p13) which was shown to be a der(19)t(2;19) in one case (21197, Fig. 2*a*) and a der(19)t(10;19) in another case (21233). In three cases diagnosed as CML the translocation t(9;22) identified by G-banding was confirmed with SKY. One case of CML (21232) was apparently Ph- by G-banding and the translocation t(9;22) was also not detected by SKY. Reverse transcriptase PCR (RT-PCR) revealed the presence of a *BCR–ABL* fusion transcript.

Five cases (21500, 19990, 21646, 21050 and 21571) had very complex rearrangements; whereas some of the involved chromosomes were identified as abnormal or missing with standard cytogenetics, the precise nature of the abnormality was only revealed using SKY. In case 21500, for example, the abnormalities identified initially included a marker and a ring chromosome. SKY analysis revealed a complex translocation/inversion involving chromosomes 4 and 5 (Fig. 2*d*). The small marker and the ring chromosome were identified as containing chromosome 7 and 12 material. For case 19990, the abnormalities identified with G banding were interpreted as an add(16p),der(19)t(15;19),-20,+mar1,+mar2. SKY, however, modified this interpretation to a der(16)t(15;16), and a der(19)ins(19;20). One marker was a chromosome 19 with deletion of both long and short arms (Fig. 2*e*) and the second marker was derived from the rearranged chromosome 20.

The karyotype in case 21646 with -5,del(13),-16,+2mar became a del(5q), a translocation der(13)t(13;13), and del(16q) (Fig. 2*f*). Case 21571 (Fig. 2*g*) had an add(5p),del(6q), add(17q); the

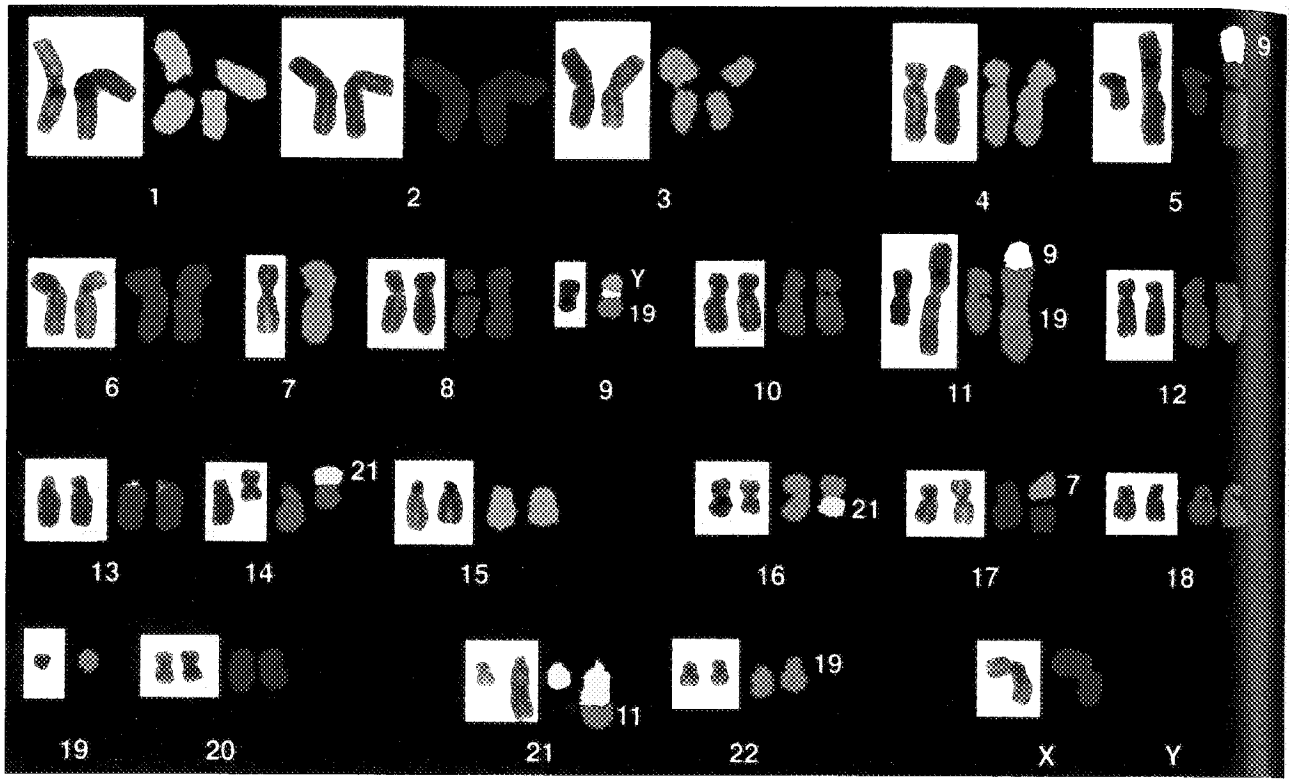

Fig. 3 Cytogenetic analysis of the cell line established from a patient with ALL (20955). Karyotype display of chromosome banding and SKY analysis of the same tumour cell. The numbers next to the aberrant chromosomes indicate the origin of translocated material. Numerous chromosomal aberrations including large marker chromosomes with complex rearrangements could be detected and characterized unambiguously.

add(5p) and del(6q) were an apparently balanced translocation involving chromosomes 5 and 6 with the derivative chromosomes composed of both long arms (add 5p) or both short arms (del(6q)). The add 17q was shown to be a duplication of 17q, likely involving the chromosomal region q23 to q25. For case 21050 (Fig. 2*h*), the translocation t(1;4) was confirmed by SKY, the del(6q) was identified as a translocation of part of chromosome 18 onto the deleted chromosome 6 so that more of 6q was lost than was determined initially, and an add(15) was identified as a der(15)t(2;15). In this case and in one other (21571, Fig. 2*g*), chromosomes thought to be normal were shown to have undetected translocations. For case 21571, a 'normal' 9 was shown to be a der(9)t(6;9). For case 21050 a 'normal' 8 had the terminal band of the long arm replaced with material derived from chromosome 3; therefore the translocation t(3;14) was shown by SKY to involve chromosomes 3, 8, and 14. In case 21232 (Fig. 2*b*) karyotype analysis by G-banding identified the t(6;8;7), but SKY permitted the assignment of the breakpoints more precisely. The case was originally selected because the identity of material added to chromosome 21 was unknown and SKY determined that it was derived from chromosome 2.

One cell line (20955), established from a patient with ALL was included in this study. The karyotype was highly rearranged which prevented a complete G-banding analysis. SKY permitted the identification of all marker chromosomes and the complete karyotype description (Fig. 3). Notably, a der(9)t(9;19) was shown to be a three-way translocation involving chromosomes 9, 19, and the Y chromosome, and a dicentric marker chromosome dicdup(11)t(9;11) was shown to contain material from chromosomes 9, 11, and 19. One marker chromosome was derived from chromosome 19 material, and a second marker contained chromosome 14 and 21 material (Table 1).

In one case with a small marker chromosome (21201), we were unable to assign the origin of the marker to a single chromosome; the SKY results, however, suggested that the marker was derived from either chromosome 2 or 12. Conventional dual-colour FISH with chromosome 2 and 12 specific painting probes showed that the marker contains chromosome 12 material.

Although determining the origin of previously unidentified chromosome material has been a major result of this study, clarification of chromosomal breakpoints is also very important. Many leukaemia and lymphomas have recurring chromosome deletions including 5q, 7q and 20q in myeloid leukaemia and 6q in lymphoid leukaemia and lymphomas. Analysis of the minimal region of deletion is a critical step in identifying and cloning the involved genes[8]. SKY has helped to correct the breakpoints originally assigned in the G-banding analysis. In case 20794 (Fig. 2*c*) for example, the abnormal chromosome 7 was thought to be a normal 7 to band q34 with material added to the end of the long arm. SKY showed that it was an unbalanced translocation with chromosome 14 and the break on 7q was likely at 7q22 with deletion of distal material. A deletion of 7q22 to q24 has been shown to be the most common region of 7q deletions in patients with AML[8]. Therefore, rather than being an exception, this patient becomes useful for further genetic analysis. Similarly for case 21050, with non-Hodgkin lymphoma (NHL), a del(6)(q22q27) was shown to be an unbalanced translocation with chromosome 18 with a deletion of 6q involving a break in 6q21 (Fig. 2*h*).

In 15 cases of haematological malignancies with complex chromosomal rearrangements, SKY analysis resulted in the elucidation of previously unidentified chromosomal material, as well as confirming all of the numerical chromosomal aberrations detected by G-banding analyses. In all instances where G-banding classified a chromosome as add(x), the additional chromosomal material was identified and the karyotype interpretation modified. SKY failed to delineate unambiguously the chromosomal origin of a marker chromosome in a case of MDS (21201) due to the small size of the marker. Spectral classification of the marker chro-

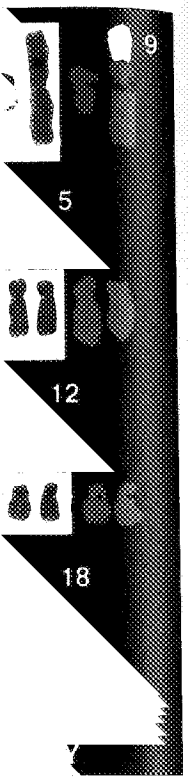

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Table 1 • Comparison of karyotype results from G-banding and SKY analyses for 15 patients

Case No.	Age	Sex	Diagnosis	G-Band Karyotype	SKY Karyotype
21197	52	M	CML	46,XY, t(9;22)(q34;q11), **add(19)(p13)**[20]	46,XY, t(9;22), **der(19)t(2;19)**[4]
21233	62	M	AML-M6+CML?	47,XY, t(9;22)(q34;q11), del(10)(p13p15), **add(19)(p13)**, **+add(19)(p13)**[11] *46,XY,t(9;22)(q34;q11)* [9]	47,XY,t(9;22), del(10), **der(19)t(10;19)**, **+der(19)t(10;19)**[1] *40,XY,der(9)t(9;22),broken* [2]
20818	49	F	CML	46,XX, t(9;22)(q34:q11), **add(11)(q23.3)**, t(14;15)(q22;q13)[20]	46,XX, t(9;22), **t(3;11)(q26;q23.3)**, t(14;15)[3]
21232	53	M	CML,accelerated phases	46,XY, inv(7)(p11q31) t(6;8;7)(q23;q23;p15), **add(21)(q22)**[2] *46,XY* [2] *46,XY,inv*(7)[14] *46,idem,t*(6;8;7) [6]	46,XY, inv(7), t(6;8;7), der(21)t(2;21)[3]
21272	57	F	AML-M7&DS	47,XX, **add(7)(p11)**, +21c[12] *47,XX,+21c* [8]	47,XX, **del(7p)**, +21[3]
20794	63	M	AML&MPD	45,X, t(Y;12)(q11.2;p11.2), -5, **add(7)(q34)**, +8, -14[19]	45,X, t(Y;12), -5, **der(7)t(7;14)(q22;q1)**, +8, -14[2]
21201	57	F	MDS	47,XX,inv(9)(p11q13)c, **+mar**[20]	47,XX,9qh+, **del(12)(p-q-)**[6]
21500	68	M	MDS	46,XY, **add(3)(p13)**, **-5**, dic(7;12)(q11;p11 or p12), **+mar** **+r**[10] 45,idem,-r[8] *46,XY*[2]	46,XY, **del(3)(p13)**, **-4,der(5)t(4;5)inv(5)**, der(7;12), **+der(7;12)**, **+r(7;12)**[3] 45,idem,-r[2] *46,XY*[2]
19990	68	M	MDS(Refractory anemia with excess blasts-RAEB-T)	**45**,XY,del(5)(q13q33),-7,-15, der(8)t(3;8)(q21;q24), **add(16)(p13)**, **der(19)t(15;19)(q13;q13)**, -20, **+mar1**, **+mar2**[12] **45**,idem,**t(7;16)(p1?4;q24)**[2] *46,XY* [6]	**44**,XY,del(5),-7,-15, der(8)t(3;8), **der(16)t(15;16)**, **der(19)ins(19;20)**, -20, **+del(19)(p-q-)**[4] *45,idem,der(18)t(7;18)*,**del(20)**[1] **44**,idem,**t(7;17),ins(16;17)**[1]
21646	64	M	t-MDS&Cancer	45,X,-Y, t(1;1)(p22;q32), **-5, del(13)(q12q14)**, **-16,+mar1**, **+mar2**[18] 45,X,-Y,**-5,+mar1**[2]	45,X,-Y, t(1;1), **del(5)**, **del(16)**, **der(13)t(13;13)**[4] 45,X,-Y,**del(5)**[1]
21571	24	M	ALL	45,X,-Y, t(2;10)(q2?1;q2?2), **add(5)(p14),del(6)(q21q25)**, t(12;22)(q13;q13), **add(17)(q25)x2**[19] *46,XY* [5]	45,X,-Y, t(2;10), **t(5;6)**, **der(9)t(6;9)**, t(12;22), **dup(17q)x2**[3]
20801	23	M	ALL	46,XY, t(2;10)(p13;p13), **add(17)(p11)** [18] *46,XY* [2]	46,XY, t(2;10), **del(17p)**[2]
21114	5	M	ALL	60,XY,+X,+Y, +4x2, **+8**, **+10x2**, add(12)(p13), +14x2, +18x2, **+?18**, +21x2[2] *59,idem,-?18* [15] 46,XY[3]	60,XY,+X,+Y, +4x2, **+8x2**, **+10**, **dup(12p)**, +14x2, +18x2, **+der(10)t(10;13)**, +21x2[2] *60,idem,-4,+der(4;17)* [1] *60,idem,-der(10)t(10;13),+10* [1] 46,XY[1]
20955	4	M	ALL	**43**,X,-Y, **add(5)(q13)**, der(5)t(5;9)(p13;q?13), -7,-9, **der(9)t(9;19)(q11;q11)**, **dicdup(11)t(9;11)**, der(16)t(16;21)(q?13;q?22), der(17)t(7;17)(p14;p12), -19 **-19,+mar1** **-21,+der(?)t(?;16;11)**, **+mar2**[6]	**42**,X,-Y, **del(5)**, der(5)t(5;9), -7,-9, **der(9)t(Y;9;19)**, **dicdup(11)t(9;11;19)**, -14, der(16)t(16;21), der(17)t(7;17), -19 **del(19)**, **der(21)t(11;21)dup(21)**, **der(?)t(14;21)**, **der(22)t(19;22)**[6]
21050	64	M	NHL	49,XY,+X, **t(3;14)(q27;q32)**, der(4)t(1;4)(q12;q35), **del(6)(q22q29)**, +7, +12, **add(15)(p11)**[19] *46,XY* [1]	49,XY,+X, **der(8)t(3;8),der(14)t(8;14)**, der(4)t(1;4), **der(6)t(6;18)(q21;q21)**, +7, +12, **der(15)t(2;15)**[2]

Table footnote: Case number, age, gender, and diagnosis of each patient is shown (CML, chronic myelogenous leukaemia; AML, acute myelogenous leukaemia; MPD, myelo-proliferative disorder; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma; DS, Down syndrome). Karyotype description follows standard nomenclature. Bold type denote aberrations refined by SKY analysis. In some instances certain karyotypes were detected only by G-banding or only by SKY which is due to clonal heterogeneity. These clones are listed in italic type.

mosome suggested either chromosome 2 or 12 material. The marker was definitively identified after dual-colour FISH using chromosome 2 and 12 painting probes as being derived from chromosome 12. One aberration that was described by G-banding but not by SKY involved a t(3;14) in case 21050. This translocation was shown to have a more complex pattern. In fact, the terminal band of chromosome arm 8q was replaced by chromosome 3 material. In addition, chromosome 14 showed chromosome 8 material translocated to its long arm. This rearrangement does not appear to be a three-way translocation because chromosome 14 material was not found on chromosome 3.

This study was designed to evaluate the feasibility of using SKY on chromosome preparations from haematological malignancies on a routine basis rather than as an attempt to identify recurrent chromosomal aberrations in a specific tumour. However, in two of four cases of CML a marker chromosome previously identified as an add(19) could be characterized by SKY as a translocation involving chromosome 19 and chromosomes 2 and 10. It is noteworthy that the t(12;21) involving *TEL* and *AML1* — despite its being the most common translocation (25%) in childhood B-cell ALL — was only discovered in 1994 (ref. 9). It is conceivable that the analysis of an increased number of cases of the same tumour entity will reveal hitherto unrecognized recurring chromosomal aberrations which involve the exchange of material with a similar banding pattern. This information should improve differential diagnosis based on the refined karyotype analysis. The reconstruction of complex chromosomal aberrations by SKY will also provide insight into mechanisms of chromosomal aberrations and clonal evolution.

SKY has the potential to become an important molecular cytogenetic technique for the identification of subtle translocations, marker chromosomes and the delineation of complex chromosomal aberrations without requiring any preconceived notion of the abnormalities involved. It is clear that the major strength of hybridization based karyotyping techniques — SKY or m-FISH[10] — remains the detection of aberrations that change the colour of the aberrant chromosome, such as translocations or insertions, and the identification of marker chromosomes. Because of the nature of chromosome painting probes, small duplications, deletions and paracentric or pericentric inversions are more difficult to detect using chromosome painting alone. Therefore, more precise cytogenetic diagnosis requires a combination of chromosome banding and SKY on the same tumour cell[6]. Future developments are directed towards semi-automated or automated microscope equipment and karyotyping software that allows for the rapid evaluation of a large number of metaphase cells by both G-banding and SKY and the evaluation and integration of the results of the respective methodologies.

Methods

Patient data and karyotype analysis: Patient samples were obtained with informed consent and the karyotype analysis was performed at the University of Chicago using standard techniques[11]. The diagnoses based on the FAB-classification and karyotype interpretations are presented in Table 1. Metaphase chromosomes for G-banding were prepared from 24- and 48-h cultures. In one case (20955) metaphase chromosomes were prepared from an established cell line.

Spectral karyotyping: Slides for SKY were freshly prepared using chromosome suspensions that were stored in methanol/acetic acid for up to two years at –20 °C. SKY was performed without prior knowledge of the chromosomal aberrations as identified by G-banding in each case. On average 5 metaphases were analysed by SKY. Low mitotic indices in the tumours, however, prevented in some cases the analysis of 5 cells. Chromosome painting probes were generated from flow sorted human chromosomes using sequence independent DNA amplification[12]. Chromosome labeling was performed by directly incorporating Spectrum Green-dUTP, Spectrum Orange-dUTP, Texas Red-dUTP, biotin-16-dUTP, and digoxigenin-11-dUTP (Vysis, Molecular Probes, Boehringer Mannheim) in a secondary PCR reaction as described[6]. Briefly, 200 ng of each of the differentially labelled chromosome painting probe was precipitated in the presence of 50 µg of the Cot-1 fraction of human DNA (BRL). Hybridization took place for 2 days at 37 °C. The biotinylated probe sequences were visualized using avidin Cy5 (Amersham Life Sciences), and the digoxigenin-labelled probe sequences by incubation with an anti-mouse digoxin antibody (Sigma Chemicals) followed by a goat anti-mouse-antibody conjugated to Cy5.5 (Amersham Life Sciences). Chromosomes were counterstained with DAPI and covered in paraphenylene-diamine (Sigma).

Image acquisition was performed using a SD200 Spectracube (Applied Spectral Imaging, Inc.) mounted on a Leica DMIRBE microscope using a custom designed optical filter (SKY-1, Chroma Technology, Brattleboro, VT). Using a Sagnac interferometer in the optical head, an interferogram was generated at all image points that is deduced from the optical path difference of the light which in turn depends on the wavelength of the emitted fluorescence. The spectrum was recovered by Fourier transformation[13,14]. The spectral information was displayed by assigning red, green or blue colours to certain ranges of the spectrum. This red, green, blue (RGB)-display renders chromosomes that were labelled with spectrally overlapping fluorochromes or fluorochrome combinations a similar colour (Fig. 1*b*). Based on the measurement of the spectrum for each chromosome, however, a spectral classification algorithm was applied that allows the assignment of a pseudocolour to all points in the image that have the same spectrum. This algorithm forms the basis for chromosome identification by spectral karyotyping (Fig. 1*c*). DAPI images were acquired from all metaphases analysed using a DAPI specific optical filter.

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